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Curcumin reverses breast tumor exosomes mediated immune suppression of NK cell tumor cytotoxicity

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Abstract

An important characteristic of tumors is that they at some point in their development overcome the surveillance of the immune system. Tumors secrete exosomes, multivesicular bodies containing a distinct set of proteins that can fuse with cells of the circulating immune system. Purified exosomes from TS/A breast cancer cells, but not non-exosomal fractions, inhibit (at concentrations of nanograms per ml protein) IL-2-induced natural killer (NK) cell cytotoxicity. The dietary polyphenol, curcumin (diferuloylmethane), partially reverses tumor exosome-mediated inhibition of natural killer cell activation, which is mediated through the impairment of the ubiquitin–proteasome system. Exposure of mouse breast tumor cells to curcumin causes a dose-dependent increase in ubiquitinated exosomal proteins compared to those in untreated TS/A breast tumor cells. Furthermore, exosomes isolated from tumor cells pretreated with curcumin have a much attenuated inhibition of IL-2 stimulated NK cell activation. Jak3-mediated activation of Stat5 is required for tumor cytotoxicity of IL-2 stimulated NK cells. TS/A tumor exosomes strongly inhibit activation of Stat5, whereas the tumor exosomes isolated from curcumin-pretreated tumor cells have a lowered potency for inhibition of IL-2 stimulated NK cell cytotoxicity. These data suggest that partial reversal of tumor exosome-mediated inhibition of NK cell tumor cytotoxicity may account for the anti-cancer properties of curcumin.

Keywords: Breast tumor exosomes; Ubiquitination; NK immunosuppression; Curcumin

1. Introduction

The identification of tumor antigens and subsequent production of tumor-specific antibodies is of special interest to many oncologists for both tumor detection and as a means for targeting the tumor with therapeutic agents [1–7]. This, of course, begs the question of why the host immune system does not recognize the tumor in the first place or loses the capacity to recognize the tumor? Recently, the idea that cancer may represent a failure of the immune system has re-emerged [1,3]. A potential mechanism for this is the secretion of a specific vesicular fraction (exosomes) by the tumor that fuses with natural killer (NK) cells and T cells and inhibits their responses to activation, thereby creating an immune tolerance [8]. This mechanism permits a scenario whereby the loss of defense to the tumor can occur at any time, perhaps related to regulation of the amount and composition of the exosomes by the tumor and other external factors.

Exosomes represent a population of membrane vesicles that are homogenous in size (ranging from 60 to 100 nm) and shape. They are formed initially by inward budding of the limiting membrane into the lumen of endosomes, creating multi-vesicular endosomes. The exosomes are most likely secreted into the extracellular environment upon fusion of the multi-vesicular endosomes with the plasma membrane [9]. A recent study has demonstrated a role for exosomes in the modulation of immune functions [8]. Exosomes from the serum of pregnant women can suppress the expression of important T-cell signaling components including CD3-ξ and JAK3 [54]. Exosomes were also shown to play a role in the control of tumor growth. Pretreatment of mice with exosomes derived from murine mammary carcinomas

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augmented subsequent tumor growth by inhibiting the cytolytic activity of NK cells [8].

The anticancer potential of curcumin in various systems has been studied extensively [10–15]. Curcumin has been shown to block transformation [16], tumor initiation [17–19], tumor promotion [20–23], invasion [24–26], angiogenesis [27–30], and metastasis [11,31–33] through a variety of mechanisms including regulation of transcription factors such as suppression of NF-κB, AP-1, and beta catenin activation [34–37]; blocking proteasome activity [38–42]; and inhibition of the cell cycle [26,43–46]. Here, we show that curcumin reverses tumor exosome-mediated inhibition of NK cell cytotoxicity. Curcumin treatment of tumor cells leads to enhancement of ubiquitination of exosomal proteins, further reducing their biological activity to inhibit NK cell tumor cytotoxicity.

2. Materials and methods

2.1. Cell culture

The TS/A cell line, a moderately differentiated and immunogenic murine mammary adenocarcinoma of spontaneous BALB/c origin (MHC class I^+, H-$2D^d$, H-$2K^d$) was maintained *in vitro* at 37 °C in a humidified 5% CO_2 atmosphere in air in complete medium (DMEM with 5% FCS) as described previously [8]. B16 melanoma cells were obtained from the ATCC and maintained in the ATCC-recommended media supplemented with 10% FBS. 4T.1 murine mammary tumor cells were a gift from Dr. Theresa V. Strong (University of Alabama at Birmingham), and were cultured in DMEM media supplemented with 10% FBS.

NK cells were prepared by depletion of splenocytes of all other cell types via MACS beads (NK isolation kit, Miltenyi Biotech, Auburn, CA) with a typical yield of >90% CD49b-positive NK cells. NK cells were then cultured in IMDM supplemented with 50 μM 2-ME, 10% heat-inactivated FCS in the presence of recombinant IL-2 (100 U/ml, R&D Systems, Minneapolis, MN) before using.

2.2. Polyphenols

Polyphenols including curcumin (diferuloylmethane), genistein (5,7,4′-trihydroxyisoflavone), quercetin (3,5,7,3′,4′-pentahydroxyflavone), calycosin, and biochanin A (7,3′-dihydroxy-4′methoxyisoflavone) were purchased from Sigma (St. Louis, MO). Baicalein (5,6,7-trihydroxyflavone) was purchased from Cayman Chemical Company (Ann Arbor, Michigan). All stocks were dissolved in DMSO and stored at −20 °C until used.

2.3. Preparation of exosomes

For preparation of exosomes, TS/A, 4T.1, and B16 cells were cultured *in vitro* at 37 °C in a humidified 5% CO_2 atmosphere in air in complete medium (DMEM with 5% FCS that had been centrifuged for 16 h at 141,000×g_{max} to exclude bovine exosomes). The supernatants were harvested from these cells after 48 h in culture. The exosomes were purified from the supernatants by differential centrifugation as described previously [8]. After differential centrifugation, exosomes were sedimented at the final centrifugation step and were resuspended in PBS and re-sedimented at 70,000×g_{max}. Sedimented exosomes were resuspended in 5 ml of 2.6 M sucrose, 20 mM Tris–HCl, pH 7.2, and floated into an overlaid linear sucrose gradient (2.0–0.25 M sucrose, 20 mM Tris–HCl, pH 7.2) in an SW41 tube for 16 h at 270,000×g_{max} to remove non-membranous protein (complexes). Gradient fractions (1 ml) were collected via the bottom of the tube and washed with PBS by centrifugation at 70,000×g_{max} for 1 h. Finally, the exosomes (fraction 3) were resuspended in PBS. Fractions 6 and 7, which contained non-membrane protein complexes, were collected and concentrated using a protein concentrator with a 100-kDa cutoff (100 kDa MWCO, Amicon). Fractions 6 and 7 were used as an exosome control (E-control). The protein content of the exosome and E-control was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). Aliquots of each fraction were stored at −80 °C before examination for the presence of exosomes by electron microscopy.

2.4. NK cell proliferation assay

NK cell proliferation was determined using a ^{3}H-thymidine incorporation assay as described previously [47]. In brief, NK cells (1×10^4/well in 96-well plates) were stimulated with IL-2 (100 U/ml) for various times with or without tumor exosomes. Plates were then pulsed with 1 μCi of [^{3}H]-thymidine (NEN, Boston, MA) and harvested after 14 h onto glass-fiber mats; incorporation of radiolabeled thymidine into DNA was determined using a scintillation counter.

2.5. NK-cell cytotoxicity

Spleen NK cells were dispensed into a 96-well microtitre plate. Cells were cultured in the RPMI1640 supplemented with 10% FBS and IL-2 (100 U/ml) for 1 to 3 days in the presence of different concentrations of tumor exosomes. Specific NK-cell cytotoxic activities were determined using a standard 4 h chromium release assay as described previously [47]. Briefly, spleen NK cells were plated in triplicate in 96-well U-bottom culture plates (Corning Glass, Corning, NY) and co-cultured for 4 h with sodium ^{51}Cr-chromate-labeled (100 μCi; NEN, Boston, MA) TS/A tumor cells. Supernatants were collected, radioactivity measured, and the specific lysis calculated according to the following equation: percentage of specific cytotoxicity=(experimental cpm−spontaneous cpm)/(maximum cpm−spontaneous cpm)×100. Maximum chromium release was determined from supernatants of lysed target cells incubated with Triton X-100 (5% v/v). Spontaneous release was determined from target cells incubated without added effector cells.

2.6. Immunoblot analysis

Western blot analysis of proteins expressed in NK cells treated with tumor exosomes was carried out using a method as described previously [8]. In brief, NK cell lysates were boiled in SDS sample buffer, and 50 μg of total protein was loaded into each well of an SDS-gel for separation by PAGE. The proteins were then transferred onto nitrocellulose membranes, and the blotted membranes blocked for 1 h with PBS-Tween 20 (0.25 M Tris, pH 7.5, PBS, 150 mM sodium chloride, and 0.2% Tween 20) containing 5% BSA. Blots were then probed overnight with 1 μg/ml of a primary Ab, washed three times with PBS-Tween 20 and then probed for 1 h with the appropriate Alexa Fluor 680 (Molecular Probes) or IRdye 800 (Rockland Immunochemicals). conjugated secondary Ab. After three washes with PBS-Tween 20, blotted proteins were detected and quantified using an Odyssey infrared imaging system (LI-COR, Lincoln, NE). The following antibodies were used for the western blots: anti-multiubiquitinated protein monoclonal antibodies FK2 (dilution, MBL, Woburn, MA), β-actin (1:5000, Sigma, St. Louis, MO), Jak3 and Jak1 (1:1000; BD Pharmingen, San Diego, CA), and phosphorylated Stat5 (1:500; BD Pharmingen, San Diego, CA).

2.7. Labeling TS/A exosomes

The PKH67 kit was used for labeling TS/A tumor exosomes according to the manufacturer's instructions (Sigma). The protocol has been described in detail previously [8]. In brief, 1 μg of TS/A tumor exosomes in 100 μl of PBS were resuspended in 1 ml Diluent C. The exosomes were mixed rapidly with a freshly prepared PKH67 solution in Diluent C (final concentration during labeling step: 5×10^{-6} M) and incubated for exactly 3 min to ensure homogeneous staining. The labeling step was stopped by addition of an equal volume of FBS for 1 min, followed by an equal volume of complete DMEM medium. After three washes in PBS by ultracentrifugation, the exosomes were resuspended in 100 μl of complete culture medium containing FBS and enumerated. All the staining procedures were carried out at 22 °C.

2.8. Internalization of exosomes by NKs

TS/A exosomes were labeled with PKH67 as described above, mixed with 1×10^6 spleen NK cells (30 min at 37 °C). The uptake of exosomes by NK cells was stopped by washing in cold PBS, transferred onto a glass slide, followed by fixation in PF and determination of TS/A exosomes transfection efficiency through visualization using a Leica TCS-NT confocal microscope (Leica Microsystems, Deerfield, IL). To quantify the efficiency of exosomes uptaken by NK cells, five fields were randomly selected on each slide, and the average number of green fluorescent positive NK cells was determined by adding the total number of green fluorescent positive NK cells, then dividing by total number of cells in five fields.

2.9. Statistics

All results are expressed as the mean ± SEM. The statistical significance was assessed using one-way ANOVA with the Bonferroni correction.

3. Results

3.1. Tumor exosomes inhibit IL-2 stimulated NK cell tumor cytotoxicity

To determine which fraction collected from the sucrose-gradient had inhibitory activity on NK cell functions, each fraction was analyzed for its inhibition of the proliferation of NK cells isolated from BALB/c mice and tumor cytotoxicity against TS/A tumor cells. The effects of different fractions were compared with those elicited by an exosomes-depleted fraction (E-controls). Fraction 3, containing exosomes as shown by electromicroscopy (Fig. 1A, insert), strongly inhibited the proliferation of IL-2 stimulated NK cells in comparison with other fractions (Fig. 1A, $P<0.001$). The results of the 4 h ^{51}Cr release assay also indicated that fraction 3 prevented IL-2 activated NK cells from killing ^{51}Cr labeled TS/A tumor cells (Fig. 1B). Similar results were obtained with exosomes of 4T.1 breast tumor cells (Fig. 1C) and B16 melanoma tumor cells (Fig. 1D).

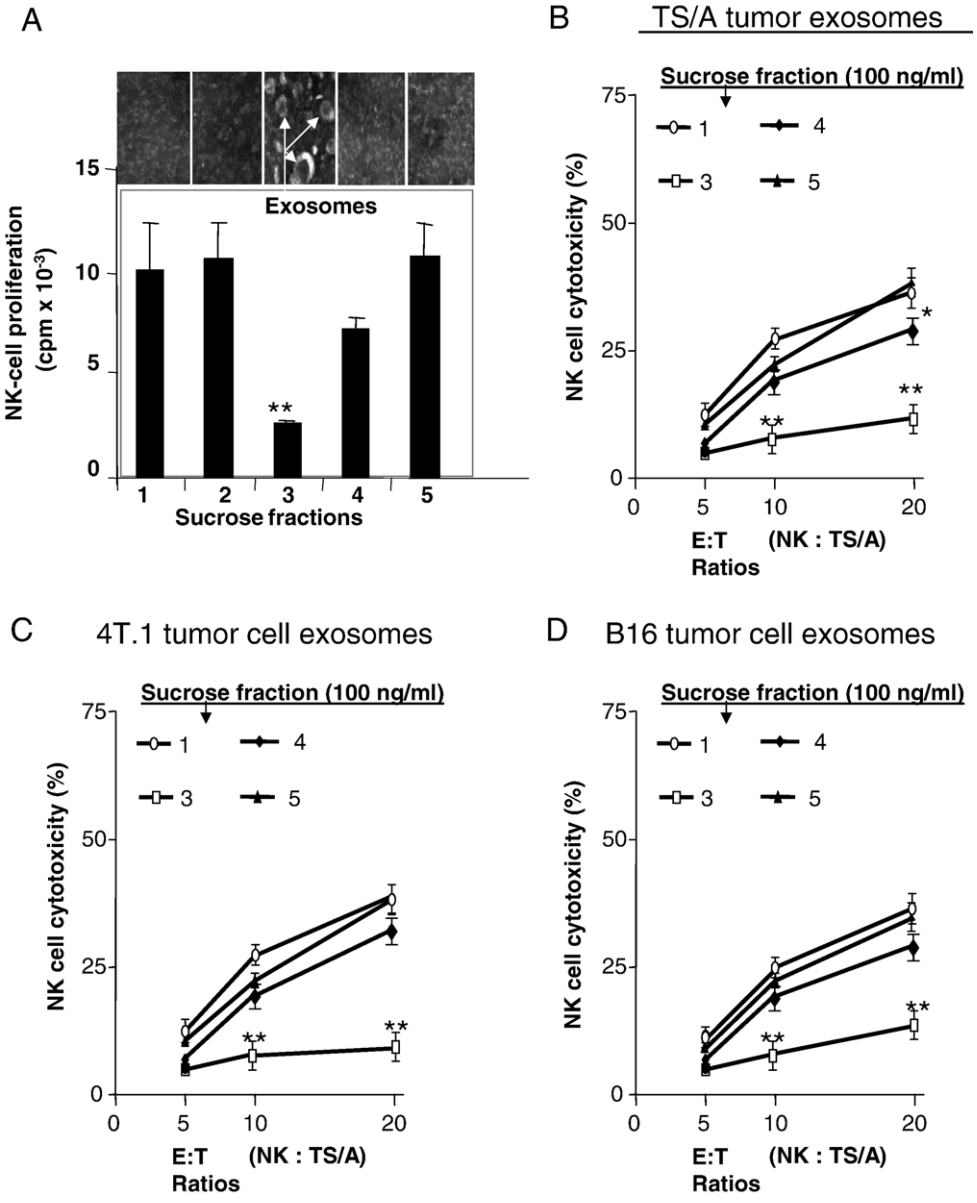

Fig. 1. TS/A breast tumor exosomes inhibit IL-2 stimulated NK cell activation and tumor cytotoxicity. Splenic DX5$^+$ NK cells were cultured with IL-2 (100 U/ml) with different fractionated samples (100 ng/ml) harvested from sucrose gradients of TS/A supernatants prepared as described in Materials and methods. At day 3 of culture, (A) IL-2 stimulated NK T cell-proliferative response was measured using a ^{3}H-thymidine uptake assay after an 18-h pulse of ^{3}H-thymidine; or (B) NK cells were added to ^{51}Cr-labeled TS/A cells at varying effector/target ratios as indicated. The amount of ^{51}Cr released into the supernatant was determined, and the percent specific release was calculated. Data are presented as the mean ± SEM of triplicate wells representative of three independent experiments. The asterisk above the bar indicates tumor exosome treated NK cells tumor cytotoxicity that was significantly different from the E-control treated NK cells. *$P<0.05$, **$P<0.01$.

3.2. Curcumin pretreated tumor cells lower tumor exosomes suppression of NK cell tumor cytotoxicity

In the first test of the hypothesis that dietary polyphenols can reverse the inhibitory effects of tumor exosomes on NK cell tumor cytotoxicity, five different polyphenols (1 μM) were examined. Thirty-six h after addition of each polyphenol to TS/A tumor cell cultures, the exosomes were sucrose gradient-purified and reversal of the inhibitory effect of TS/A tumor exosomes on NK cell cytotoxicity against TS/A tumor cells was examined. The NK cell cytotoxicity was calculated using a formula as described in Materials and methods. The effects of exosomes isolated from the TS/A tumor cells treated with different polyphenols or with polyphenol carrier (DMSO) at the same concentration used as the solvent in NK cell tumor cytotoxicity assays were compared with the cells treated E-control isolated from the DMSO treated TS/A tumor cells. Of these, curcumin had the strongest effects on NK cell killing ^{51}Cr labeled TS/A tumor cells although among five polyphenols tested, three polyphenols have significant effects on the reverse of tumor exosome-mediated inhibition of NK cell tumor cytotoxicity. At ratios of 20 : 1 (IL-2 stimulated NK cells : TS/A tumor cells), the percentages of tumor cytotoxicity caused by NK cells increased significantly from 5.2±1.1% (E-control) to 22.4±4.2% ($P<0.01$), 14.8±3.8% ($P<0.05$), 10.2±1.8% ($P<0.05$), and 6.70±0.6% ($P>0.05$) when TS/A exosomes purified from TS/A tumor cells pretreated with curcumin, baicalein, genistein, and DMSO (carrier control), respectively. Other polyphenols including biochanin A and quercetin tested had no significant effects on TS/A tumor exosome-mediated inhibition of NK cell tumor cytotoxicity (data not shown). Use of a wider range of concentrations revealed that the effect of curcumin was dose-dependent and that even at 200 nM it caused a significant attenuation of TS/A tumor exosome-mediated inhibition of NK cell tumor cytotoxicity ($P<0.05$, Fig. 2A). This was also the case for reversing NK cell proliferation at two different ratios of NK:TS/A cells as indicated by an increase in [^{3}H]-thymidine incorporation into DNA (Fig. 2B, C).

These data suggest that TS/A tumor cells produce exosomes that can inhibit NK cell activation and tumor cytotoxicity. Pretreatment of TS/A tumor cells with curcumin, (200 nM and above), led to partial reversal of tumor exosomes-mediated inhibition of NK cell cytotoxicity.

3.3. Curcumin treatment enhances ubiquitination of tumor exosomal proteins

Inhibition of the proteasome activity was considered as playing a role in anti-cancer activity of curcumin [40,42,44]. Exosomal proteins are regulated by the ubiquitin pathway [48–51]; therefore, we investigated whether curcumin treatment had an effect on the ubiquitination of tumor exosomal proteins. Western blot analysis of exosomes purified from TS/A tumor cell cultured supernatants indicated that curcumin treatment of TS/A tumor cells resulted in the accumulation of ubiquitinated proteins in the exosomes. Accumulation of the ubiquitinated TS/A exosomal proteins is curcumin dose-dependent (Fig. 3A). Differences in signal intensity of ubiquitinated exosomal proteins are unlikely to be due to uneven loading of protein as there was no differences in the amounts of CD9 (one of the exosomal protein markers—see Fig. 3A, bottom panel). To determine whether the accumulation of ubiquitinated exosomal proteins as a result of curcumin treatment was associated with the enhancement of NK cell tumor cytotoxicity, NK cell cytotoxicity against TS/A tumor cells was examined. TS/A tumor exosomes were isolated from the supernatants of TS/A tumor cells treated with different concentrations of curcumin and used for pulsing primary NK cells isolated from the spleens of 2-month-old BALB/c mice. After incubation with

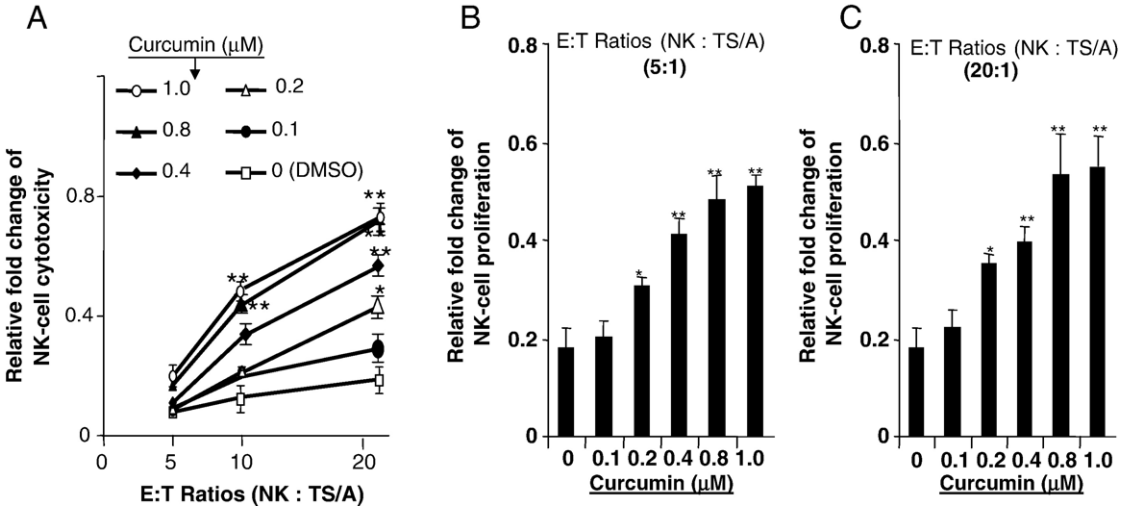

Fig. 2. Curcumin treatment of TS/A tumor cells reduces TS/A tumor exosome-mediated inhibition of NK cell activation and tumor cytotoxicity. TS/A breast tumor cells are cultured for 36 h in the presence of different concentrations of curcumin or the solvent DMSO at the same concentration as used for the highest concentration of curcumin. Exosomes were sucrose-gradient purified from the supernatants. Spleen NK cells were stimulated with IL-2 (100 U/ml) in the presence or absence of TS/A tumor exosomes for 3 days. NK cell proliferation (A), and tumor cytotoxicity of NK cells (B) were analyzed using methods as described in Fig. 1 legend. The ratios of NK cell cytotoxicity (A) or [^{3}H]-thymidine incorporation (B, C) of the cells treated with TS/A exosomes versus treated with E-control were calculated. The data are presented as the mean±SEM of five wells representative of three independent experiments. *$P<0.05$, **$P<0.01$.

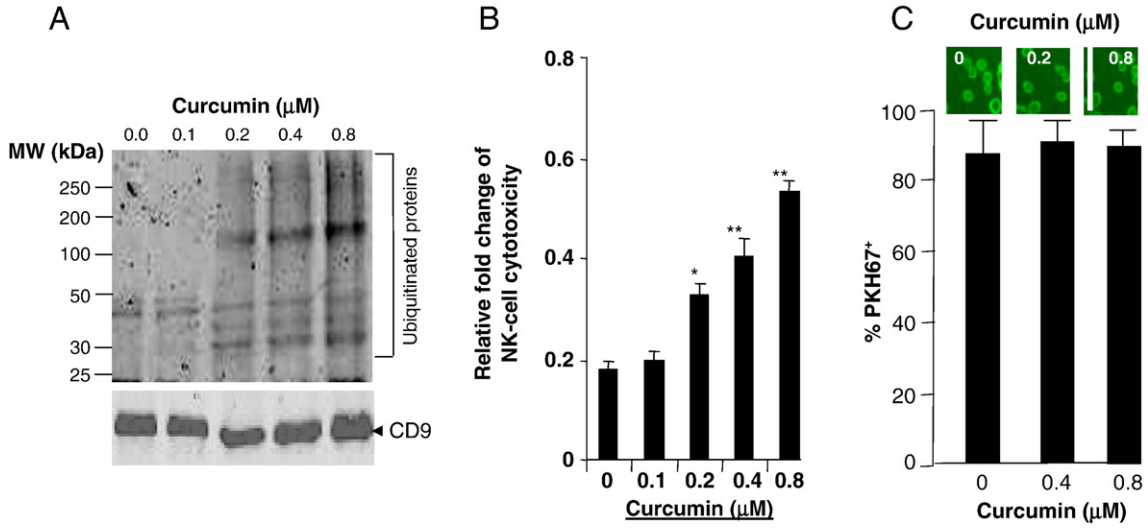

Fig. 3. Curcumin treatment of TS/A breast tumor cells increases ubiquitinated exosomal proteins without affecting the entry of exosomes. TS/A tumor cells were cultured in the presence of different concentrations of curcumin for 36 h. Exosomes were purified from the supernatants by sucrose gradient. The exosomes were lysed and analyzed for ubiquitinated proteins by western blot with a mouse anti-polyubiquitin antibody (A, the top panel), or a mouse anti-CD9, which is one of exosomal protein markers (A, the bottom panel). To determine the effects of curcumin treatment on the attenuation of TS/A tumor exosome-mediated inhibition of NK tumor cytotoxicity, spleen DX5-positive NK cells were stimulated with IL-2 (100 U/ml) in the presence of TS/A exosomes (100 ng/ml) or E-control (100 ng/ml) for 24 h. Tumor cytotoxicity of NK cells was analyzed using methods as described in Fig. 1 legend. The ratios of NK cell cytotoxicity of the cells treated with TS/A exosomes versus E-control were calculated. The data are presented as the mean±SEM of five wells representative of three independent experiments (B). TS/A exosomes were labeled with PKH67 dye as described in Materials and methods. Purified spleen NK cells (1×10^4) were stimulated with IL-2 (100 U/ml) in the presence of $PKH67^+$ TS/A exosomes (100 ng/ml) isolated from the TS/A tumor cells cultured with different concentrations of curcumin. TS/A exosome-treated NK cells were then harvested at 6 h and unbound exosomes were washed three times with PBS. The cells were centrifuged onto poly-L-lysine-coated slides and examined for green fluorescent labeled NK cells using a Leica confocal microscope: ×60 (B, insert). Percentages of $PKH67^+$ cells were calculated by counting the total number of $PKH67^+$ NK cells, then dividing by the total number of cells in five randomly selected fields. The result is expressed as the mean±SEM of the results of assays performed in triplicate. The data are representative of 4 independent experiments (C). $^*P<0.05$, $^{**}P<0.01$.

the exosomes for 24 h in the presence of recombinant mouse IL-2 (100 U/ml), the NK cells were then washed 3 times with PBS to remove unbound exosomes before the cells were used in a standard *in vitro* chromium release assay. The addition of tumor-derived exosomes strongly inhibited the NK cell-mediated killing of TS/A target cells (Fig. 3B). At a 20:1 E:T ratio, there was a 80± 7% reduction in the cytotoxic activity of the exosome-treated NK cells as compared to the NK cells that had been treated with PBS as

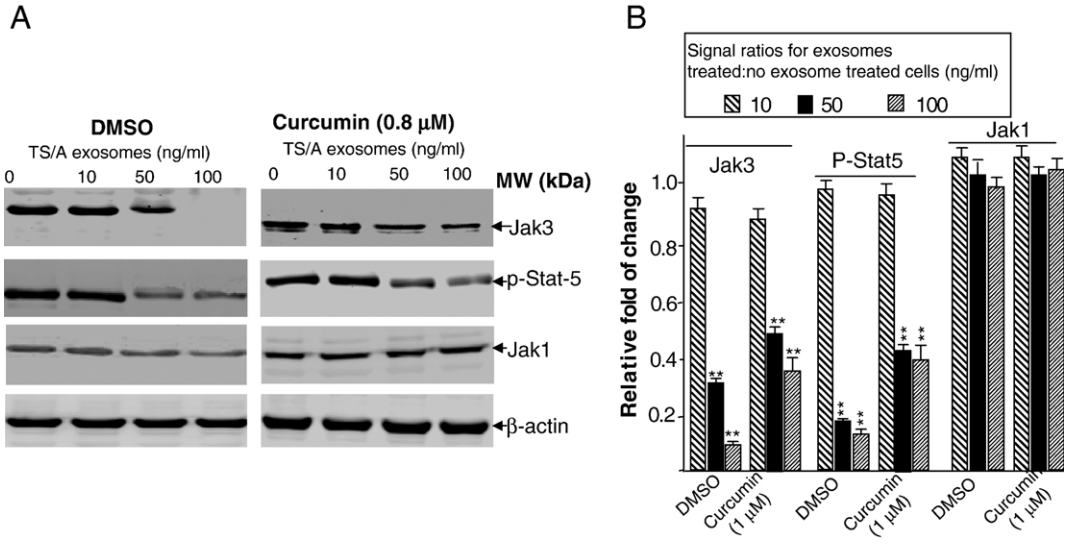

Fig. 4. Reduction in inhibition of Jak3 expression of IL-2 stimulated NK cells by TS/A tumor exosomes produced in the presence of curcumin. Spleen DX5+ NK cells were cultured in the presence of different concentrations of TS/A exosomes purified from the supernatants of TS/A cells pretreated with curcumin (0.8 μM) for 36 h. NK cells were lysed after 72 h in culture. Fifty μg of total protein from each lysate were resolved on a 10% SDS PAGE gel. After proteins were transferred to a nitrocellulose membrane, the blots were probed with the indicated antibodies (A). The signal intensity of each protein analyzed was quantified using an Odyssey infrared imaging system (LI-COR). The ratios of signal intensity of each protein tested in DMSO treated TS/A exosomes:curcumin treated TS/A exosomes were calculated and plotted (B). The data are representative of three independent experiments. $^{**}P<0.01$.

a control. In contrast, the tumor exosomes isolated from TS/A tumor cells pretreated with cucumin led to a significant attenuation of TS/A tumor exosome-mediated inhibition of NK cell tumor cytotoxicity in a curcumin dose dependent manner (Fig. 3B). The attenuation of TS/A exosomes mediated inhibition of NK cell cytotoxicity appears to be associated with the enhancement of ubiquitination of exosomal proteins.

Furthermore, the results of NK cells pulsed with PKH67 fluorescently labeled TS/A exosomes indicate that TS/A exosomes are effectively taken up by NK cells (>84±5.6%) regardless of whether TS/A tumor cells were treated with curcumin or DMSO as a carrier control (Fig. 3C). These data suggest that regardless of the treatments, NK cells have equal opportunity to uptake tumor exosomes.

3.4. Curcumin pretreatment of exosomes isolated from tumor cells lowers the inhibition of Jak3 activation in IL-2 stimulated NK cells

Our previous data suggest that expression of Jak3, but not Jak1, was inhibited by tumor-derived exosomes [8]. To determine if curcumin reversal of tumor exosome-mediated inhibition of NK cell activation and cytotoxicity was associated with the inhibition of Jak3 expression, the expression of Jak3 and Jak3 regulated molecules were quantified by western blot analysis. We found that the expression of Jak3 (Fig. 4A, the top panel), but not Jak1 (Fig. 4A, the third panel from the top), was inhibited by the tumor-derived exosomes. This inhibition of Jak3 expression was further demonstrated by a reduction in the levels of phosphorylated Stat5, which is one of Jak3 substrates (Fig. 4A, the second panel from the top). In contrast, tumor exosomes isolated from TS/A tumor cells pretreated with curcumin have a much less attenuated reduction of both the expression of Jak3 and p-Stat5. The reversal of reduction of Jak3 expression and phosphorylation of Stat5 was dependent on the concentration of curcumin used for treatment of TS/A tumor cells. The differences in the amount of phosphorylated Stat5 among samples were not attributable to differences in sample loading as equivalent levels of β-actin were detected (Fig. 4A, the bottom panel). Further quantification of expression of Jak3 and p-Stat5 indicated that upon treatment of the TS/A tumor cells with curcumin, TS/A tumor exosome-mediated inhibition of the expression of Jak3 and p-Stat5 was reversed significantly ($P<0.01$) (Fig. 4B). Pretreatment of TS/A tumor cells with curcumin had no effect on expression of Jak1 or β-actin ($P>0.05$) (Fig. 4B).

4. Discussion

The experiments in the present study have revealed that several polyphenols derived from the diet, in particular curcumin, can reverse the immunosuppressive effects of mouse mammary tumor exosomes on NK cells. To the best of our knowledge, these findings represent the first example of curcumin treatment that leads to attenuation of tumor exosome-mediated immunosuppression of NK cell activation and tumor cytotoxicity. Blocking activation of Stat5 of IL-2 stimulated NK cells is considered as one of the mechanisms underlying tumor exosome-mediated immunosuppression of NK cell activation [8]. This study suggests that function of tumor exosomes was altered after tumor cells were treated with curcumin. The exosomes isolated from the tumor cells pretreated with curcumin cause a significant reduction of immunosuppression of NK cell activation and tumor cytotoxicity. Furthermore, this reduction in immunosuppression was correlated with enhancement of ubiquitinated exosomal proteins after tumor cells were treated with curcumin. Taken together, the data are consistent with the hypothesis that diet is a factor in determining immune tolerance to tumor cells.

Curcumin, a naturally occurring polyphenolic compound found in the spice turmeric, has been reported to have many biological effects. Such a multiplicity of activities could result from the highly reactive α,β-unsaturated ketone group of curcumin. The ketone group can bind covalently to many proteins that are involved in proteasome-mediated degradation pathways [19,38,39,41,52,53] and in particular, those proteins having a E3 ligase activity. Therefore, the identification of E3 ligase(s) that are regulated by curcumin and play a critical role in tumor exosome-mediated immunosuppression should be investigated further. In addition, this initial finding as reported in this study opens up the potential strategy to identify tumor exosomal proteins that are ubiquinated and associated with curcumin treatment. These proteins most likely play an important role in tumor exosome-mediated immune suppression. We have failed to identify such proteins by mass spectrometry analysis as many exosomal proteins have become ubiquinated after curcumin treatment of TS/A tumor cells. A suitable method must be developed for addressing this issue.

Another unsolved question concerns the fact that we do not currently know whether curcumin treatment results in enhancement of the ubiquitination of house-keeping exosomal proteins and/or additional cellular proteins sorting into tumor exosomes as a result of curcumin treatment. One hypothesis is that curcumin may inhibit proteasome activity, facilitating sorting of ubiquitinated proteins into the exosomes. These proteins coexist with tumor exosomal proteins and interfere with their immununosuppression activity. Alternatively, the immunosuppressive activity of tumor exosomal proteins is inactivated once they are ubiquitinated. Further investigations will be required to address these points.

The attenuation of tumor exosome-mediated immune suppression by curcumin treatment reported here appears to be in contrast to previous reports of the ability of curcumin to suppress immune activity. Other recent studies [55] are in agreement with our study that suggests that curcumin enhances NK cell tumor cytotoxicity. The reason for the discrepancies between our study and earlier studies are numerous. Earlier reports investigated the direct effect of cucurmin on immune cells such as T cells, NK cells, and other leukocytes. However, we determined the effects of exosomes produced by tumor cells pretreated with curcumin on NK cell tumor cytotoxicity. Therefore, we did not expect to necessarily observe a similar effect on cytotoxicity when NK cells were directly treated with curcumin compared to exosomes mixed with NK cells where the exoxomes were produced from treatment of tumor cells with

curcumin. Also, discrepancies may be due to differences in the target cells used, that is, immune cells versus tumor cells, and the use of different stimuli in the experiments. For an example, curcumin treatment of K562 cells does not induce significant variations in the levels of phosphorylated Stat-5, but curcumin treatment of TS/A tumor cells leads to a reduction of p-Stat5 as observed in our work.

Our results suggest that curcumin treatment of tumor cells leads to the enhancement of ubiquitination of tumor exosome proteins, and the reduction of tumor exosome-mediated inhibition of NK cell tumor cytotoxicity. However, due to a 5–10% contamination of our NK cell populations with non-NK cells, the possibility of non-NK cells contributing to the tumor exosome-mediated inhibition of NK cell proliferation and tumor cytotoxicity via some interaction with NK cells cannot be excluded.

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